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Pyrimidine-specific ribonuclease superfamily

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Chapter 7

SUMMARY

Pyrimidine-specific ribonucleases constitute a superfamily of homologous proteins. Bovine pancreatic ribonuclease (ribonuclease A) is a typical representative of this superfamily. It can cleave RNA endonucleolytically to yield 3'-phosphomono- and oligonucleotides ending in Cp or Up, with 2',3'-cyclic phosphate intermediates. Pancreatic ribonuclease is mostly found in pancreas, but also in other organs and body fluids. Separate families with sequences that are more than 50 % identical can be distinguished in the superfamily. So far four families were found in mammals: secretory (pancreatic) ribonucleases, liver ribonucleases, angiogenins and neurotoxin-type ribonucleases. These four families were found in many sources and have arisen by gene duplications in an ancestral vertebrate. Besides the four families found in mammals, five other families were found and characterized in other vertebrates. This superfamily includes proteins that display many interesting but poorly understood biological activities, such as immunosuppressivity, cytotoxic and neurotoxic activities, antitumour activity, endothelial-cell-stimulatory activity, and lectin-like behaviour.

In this thesis we describe studies on several members of the ribonuclease superfamily, both belonging to the family of the secretory ribonucleases as other ones outside this family. Protein chemical techniques, but also a few molecular biological methods, were used for these investigations.

Two chapters describe members of the mammalian pancreatic ribonucleases. Previously several rodent ribonucleases have already been isolated and sequenced. Six of the investigated rodent species belong to the Muroid suborder, including the mole rat, *Spalax ehrenbergi*. The isolation and the sequence determination of the enzyme from the closely related species *Spalax leucodon* is described in **Chapter 2**. Only one difference with the previously determined sequence of the *Spalax ehrenbergi* was detected; the proline residue at position 42 has been replaced by alanine. Proline-42 is a well-conserved residue in mammalian pancreatic ribonuclease. The only other species with also alanine at this position is the three-toed sloth. A difference of one amino acid residue between the sequences of the two *Spalax* superspecies is reasonable in the light of their evolutionary relatedness.

In the bovine species three secretory ribonucleases are found which result from gene duplications which occurred in an ancestral ruminant species. One of the ribonucleases has been

isolated from brain tissue. Its sequence has been determined both at the protein and at the DNA level. This enzyme is expressed, besides in brain, only in lactating mammary gland. In **Chapter 3**, we describe the isolation and characterisation of this enzyme from the latter tissue. However, the enzyme could not be detected in bovine milk, using the same purification procedure. With a zymogram method, brain ribonuclease activity could be identified in lactating mammary gland but not in bovine milk. It may be concluded that bovine brain ribonuclease is not secreted into milk. An extra rather hydrophobic tail at the C-terminus of the enzyme may influence the secretion of the enzyme.

The only two non-mammalian species with a reasonable high ribonuclease content in the pancreas are two reptiles: snapping turtle and iguana. For the study of the origin of mammalian ribonucleases, reptiles are very suitable as they form a paraphyletic taxon with the birds and the mammals. Pancreatic ribonuclease from turtle pancreas had already been characterized and sequenced. Here, we describe the isolation, characterization and sequence determination of the enzyme from iguana pancreas (**Chapter 4**). The specific activity of iguana ribonuclease for RNA at pH 7.4 was similar to that of bovine ribonuclease A. The complete primary structure of iguana pancreatic ribonuclease was determined. The polypeptide chain is N-terminally blocked, consists of 119 amino acid residues, and is most similar to that of the enzyme from turtle pancreas. It has one residue less at the N-terminus and one residue more at the C-terminus. Like turtle ribonuclease it has an insertion in the S-peptide loop near residue 21, a deletion of three residues near residue 70 and a deletion of two residues near the residue at position 114 compared with mammalian pancreatic ribonucleases. Likewise, the disulphide bond near the residue at position 70 is missing. Iguana and turtle ribonuclease differ at 54 % of the amino acid positions, which implies that they belong to different families. Iguana ribonuclease contains no carbohydrate, although the enzyme possesses three recognition sites for carbohydrate attachment. It has a high number of acidic residues in a localized part of the sequence, which may explain its high apparent molecular mass of 18 kDa.

Several studies have been published about the isolation and characterization of ribonucleases from rat and mouse liver. However, structural features are still unknown. In order to assign these enzymes to one of the pyrimidine-specific ribonuclease families, or to an evolutionary unrelated one, we have started the isolation and characterization of ribonuclease from rat liver in **Chapter 5**. The presence of four members of the ribonuclease superfamily was demonstrated in this tissue. These enzymes were isolated after homogenization in dilute H_2SO_4 , acetone precipitation, gel filtration on a Sephadex G-75, and further fractionation by Sp-trisacryl column chromatography. Three fractions (RL1,

RL2 and RL3) showing ribonuclease activity at pH 7.5 with yeast RNA as substrate were isolated. Reaction with an antiserum against rat pancreatic ribonuclease showed that RL1 was identical to the rat pancreatic enzyme. Fractions RL1, RL2 and RL3 were further purified by reversed-phase HPLC on a C18 column. N-terminal sequence analysis of RL1 showed the presence of native protein and several N-terminally degraded components. RL2 and RL3 were N-terminally blocked proteins. After acidic cleavage or CNBr digestion, several parts of their sequences were determined. RL2 was a member of the neurotoxin-type ribonuclease family with high sequence similarity with RNase Us from human urine, RNase K2 from bovine kidney and RNase K3 from porcine kidney. RL3 was a very basic protein. Investigated peptide sequences are identical to those derived from a cDNA sequence of rat liver-type ribonuclease (J. Hofsteenge, personal communication), and have a high sequence similarity with ribonucleases BL4 and PL3 from bovine and porcine liver, respectively. A contaminant of the RL3 fraction had a high sequence similarity with mouse and other mammalian angiogenins.

Liver-type ribonucleases from different sources seems to prefer poly (U) as substrate above poly (C). However it was reported that bovine liver ribonuclease has not this preference. Therefore, we isolated the bovine enzyme by the methods used for rat enzyme (**Chapter 6**). Investigation of the base specificity showed that all three liver-type ribonucleases, including the enzyme from bovine liver, had a high preference of poly (U).

Through all research on ribonucleases described in this thesis, we know that the pyrimidine-specific ribonuclease superfamily consists of a group of proteins with different levels of ribonuclease activity and various special biological functions. The occurrence of a cytoplasmic ribonuclease inhibitor generally prevents cytotoxicity of ribonucleases from the same species. Although ribonucleases from the four mammalian families differ rather much in sequence, they interact with similar affinities with mammalian inhibitor. However, there are observations that this cross-reactivity does not apply for combinations of members of the superfamily and inhibitors from different vertebrate classes (mammals, birds and amphibians), which explains the cytotoxicity of the amphibian ribonuclease "onconase". These observations may give rise to the possibility of applications in medicine. For instance the reptilian ribonucleases described in this thesis (from iguana and turtle pancreas) differ rather much in sequence and substrate specificities from each other and from other members from the ribonuclease superfamily, but not more than members of the four mammalian families differ from each other. Further studies of the interactions of reptilian ribonucleases with mammalian ribonuclease inhibitors may provide interesting results and possible medical applications.